Computational modeling and experimental validation of Deformable Lipid Vesicles for Non-Invasive Delivery

A

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1. INTRODUCTION

Lipid vesicles are closed spherical self-assembled structures formed impulsively by amphipathic molecules, each having an outer hydrophilic head, long hydrophobic tail and an internal aqueous core **(Bangham et al., 1965)**. These amphipaths like macromolecules constitute numerous biological entities including membranes, micelles, and lipid droplets, and they are involved in several cellular features associated with key life processes from structure, signaling or storage **(Vattulainen and Rog, 2011).**

It has been pronounced that traditional lipid vesicles commonly restrained to the upper layer of the stratum corneum or occasionally accrued within the skin appendages with negligible diffusion to deeper tissues, as they have poor permeability and deformability **(El Zaafarany et al., 2010)**. The idea of elastic or deformable vesicles or transferosome have been proposed by Cevc and Blume in 1992 with pronounced deformability and stability **(Cevc, 1997; Cevc and Blume, 1992; Cevc, 1996, 2004).** The two major constituents in first generation deformable vesicles are phospholipids and an edge activator, the latter is classified as a single chain of surfactants whose function is to destabilize the lipid vesicle bilayer in such a way that it increases its deformability without compromising the stability **(Cevc et al., 2003; Duangjit et al., 2011**). The group of edge activators characteristically used for preparing ultradeformable vesicle includes both ionic as well as non-ionic; for instance; sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, and Tween 80. The non-ionic surfactants (edge activators) provide adaptability and flexibility in deformable lipid vesicles. The conventional vesicles fails to cross the intact mammalian skin , however deformable vesicle take advantage of minute pores (<2 nm) and owing to its flexibility as large as 50 nm-100nm size vesicles can successfully overcome the barrier of the skin, the stratum corneum (**Cevc, 1996; Cevc et al., 1998, 2002**). Consequently, they can penetrate through the intact skin for sizes up to 200-300 nm.

Deformable vesicle is dynamic, rationally designed, multicomponent vesicular system (**Cevc, 2012; Pasenkiewicz-Gierula et al., 2016)**. Most critical parameters of a lipid bilayer include rigidity and relative mobility (permeability) of the sole lipid molecules which can be mainly influenced via transition temperatures of lipid components. And thus, being the most important factor to assess

vesicle stability. All lipids have a peculiar property of phase transition i.e. the temperature at which they go through a transition change from gel to liquid phase. Lipids when kept below the phase transition temperature (Tc) are in gel phase while when the composition is kept above Tc, the lipid comes in liquid phase. Consequently, shows higher permeability across the associated molecule that's due to rapid diffusion of lipid molecules in liquid phase which were earlier constrained in gel phase.

In liquid phase these generally exist in a liquid crystalline state having a comparatively higher degree of disorder and dynamical behavior, specified as liquid disordered state. Because of this dynamic nature, its state cannot be described by any single conformation of the molecules therefore it becomes extremely difficult to study them using a range of biophysical and other techniques. Innumerable experimental assessment have been conducted over a certain time interval to evaluate the structural and dynamical properties for a large number of lipids but it not likely to occur every time that such assessment fabricate unambiguous portrait of solitary lipids and their interactions **(Lyubartsev and Rabinovich, 2016)**. To overwhelm the challenges, computational modeling and simulations taken into consideration and in fact found beneficial in providing every atomic scale detail.

The rapid accessibility of data over time causes expansion in computing power and modeling methodologies. Models allow us to make choices. They can assist us to imagine, forecast, optimize, modify, and organize composite systems. To address complex system; for instance a stable membrane, there is a surge of computational models that enable us to optimize by making selections prudently and apprehend the possible outcomes of choices (**Clader et al., 2018**). The proposed computational models for membrane design to suit the purpose are further preceded for simulation. Computational modeling, precisely employing Molecular Dynamics (MD) simulations, is a competent method to monitor the properties of membrane and to study their interactions **(Kepczynski and Róg, 2016)**. Furthermore, varying lipid molecules in various ratios is possible to analyze that may lead to precise optimization and provide a lead for experimental validation. Such an effort would be otherwise an extensive procedure through experiments **(Marrink et al., 2019; Poger et al., 2016).**

Owing to the dynamic properties of lipid vesicles offers promising potential drug and antigen delivery vehicles. Deformable vesicles are capable of eliciting both humoral and cell-mediated antibody

responses, conferring immunological protection **(Pappalardo et al., 2016**).These vesicles associates soluble as well as hydrophobic protein antigens owing to their amphipathic in nature. Further, the association of protein antigens with the lipid vesicles protects them from host cell's enzymatic breakdown and consequently broadening their half-life *in vivo* **(Henriksen-Lacey et al., 2011)**. The particulate nature of vesicles makes them better candidates to be taken up by antigen-presenting cells (APCs) for processing and presentation in conjunction with major histocompatibility complex (MHC) molecules to interact with T cell receptor on$CD4^+$ and $CD8^+$ T lymphocytes of the immune system. These vesicles also have the tendency to demonstrate depot effect that is the ability to assist long term retention and sustained launch of antigens at the route of administration. This distinctive property of vesicle gives not only T-helper2 responses and T-helper1 but also the long-term specific memory immune response (**Giddam et al., 2012**). These T_H cells along with antigen-specific B cells results in antibody production and thus neutralizing the foreign antigen, consequently leading to eradication.

The conceptual design is prerequisite for optimizing artificial vesicles to acquire optimal size, stability, permeability and deformability to achieve penetration across intact mammalian skin (essentially stratum corneum) and intact mucosal barriers. This largely depends upon three factors (i) hydrophile-lipophile balance (HLB) value of surfactant **(Bnyan et al., 2018)**, (ii) area per lipid **(Tieleman et al., 1997)**, and (iii) the permeability flux **(El Zaafarany et al., 2010).** Furthermore, the immune responses are dependent on several intrinsic properties of the vesicles, for instance; (i) size of the vesicles (ii) flexibility/elasticity (iii) composition of vesicles (iv) stability of vesicles **(Elsayed and Cevc, 2011)**. The versatility of these vesicles opens the avenues for wide applications.

The focus of this study aims at the optimization of rational vesicle composition for therapeutically immunologically successful delivery of large antigens to elicit protective immune responses. The experimentally validated computationally aided models are envisaged to conjure optimized deformable lipid membrane vesicle system for the non-invasive delivery of antigen. Such non-invasive delivered antigen either dependent on skin immune system or mucosal immune system has potential to generate protective immune responses. Till date only experiments of such lipid vesicle based non-invasive vaccine delivery has been reported **(Elsayed et al., 2007)**. The non-invasive or needleless delivery is preferred over conventional (invasive) vaccination method as these offer opportunities for

macromolecules delivery with extensive blessings along with self-medication, painless, low infection hazard and cost-effectiveness **(Bajracharya et al., 2019)**.

Model membrane simulations will ensure the precise set of formulations to be experimentally tested narrowing down (i) the number of test formulations (ii) the number of animals required to test such formulations. Thus, computational modeling enables the process to be accomplished in much less time and with knowledge-based *in silico* verified selection of the future successful formulations.

2. REVIEW OF LITERATURE:

This study converge two approaches for optimization of deformable vesicles for non-invasive delivery. Firstly, a prior analysis of interaction of components of membrane by *in silico* methods that provides promising lead as well as reduces extensive testing of number of formulations in terms of (i) variability in composition (ii) conditions in achieving deformable stable vesicles. Secondly, *in silico* modeling of deformable vesicles and their characterization through simulations. Therefore, review of literature is divided in two sections that include (1) Ultra deformable lipid vesicle and methods of optimization (2) Computational modeling of lipid vesicle.

2.1. ULTRADEFORMABLE LIPID VESICLE:

This innovation of the liposome makeup was originally made by the hematologist Alec Bangham in the mid-60s **(Bangham et al., 1965)** what he referred to as "multilamellar mesophases" or "banghasomes". The term "liposome" was coined by a visitor to his lab and appeared for the first time in a publication in 1968 (**Sessa and Weissmann, 1968**). Gregoriadis proposed their use as a drug delivery system and almost immediately foresee the potential to achieve targeted delivery. The versatility of liposomes was very quickly realized; by the end of the 70s liposomes had been suggested as a delivery mechanism for chemotherapy agents **(Gregoriadis, 1973),** drugs **(Colley and Ryman, 1975)** and enzymes **(Finkelstein and Weissmann, 1978).** Gradually, the application of liposomes was soon realized in vaccine development working as an adjuvant **(Rooijen and Nieuwmegen, 1980)**.

Despite various reported research efforts on traditional liposomes, clinical and commercial success of those vesicles with non-invasive delivery could not be achieved. This is due to the lack of rational design of liposomes that enables the skin permeation across intact biological membranes. Owing to its large vesicle size and absence of elasticity the penetration becomes nearly impossible into the skin

tissue, but rather remain in stratum corneum (**Barry, 1983**). Furthermore, scalability of the manufacturing process, chemical instability, residual organic solvent inside the drug product, liposomes aggregation, cost of lipids, and regulatory implications additionally pose extra challenges in liposomes.

To overcome the drawbacks of conventional liposomes, in 1992, Cevc and Blume were first credited with the introduction of ultra-flexible vesicle, consisting of a soya phosphatidylcholine (PC) and a non-ionic edge activator, as a carrier for transdermal delivery and it represents the first generation of ultra deformable vesicles. Owing to its flexibility the vesicles easily penetrate the biological skin barrier (stratum corneum) due to the generation of "osmotic gradient" in transdermal tissues i.e. the evaporation of water from the skin surface. The trans-epidermal hydration provides the driving force for the transport of the vesicles resulting in getting over the epidermis to reach systemic circulation (**Cevc and Blume, 1992**). Delivery of large antigens like bovine serum albumin **(Paul et al., 1995)**, insulin like drugs **(Cevc, 1996; Cevc et al., 1998**), gap junction protein **(Paul et al., 1998)** was successful in immunization across intact mammalian skin. The abilities of diverse vaccine carriers such as colloids, liposomes, micelles, deformable vesicles and mixed deformable vesicles were tested to conquer skin barrier **(Cevc et al., 2003; Cevc, 2004).**

Continuing interest in using long-circulating deformable vesicles in drug delivery is supplemented by their potential use for other purposes, such as carrying imaging agents and the treatment of infection. In a fascinating study, skin permeation and skin deposition properties of both ketoprofen fumarate-loaded deformable vesicles (phosphatidylcholine: Tween 80 ratio of 84.5:15.5 wt/wt) and conventional liposomes were studied and the former found to be 5 times more skin permeable **(Elsayed et al., 2007**). The distribution of long lasting drug NSAIDs, ketoprofen into peripheral target tissues through carriers like ultradeformable vesicles or conventional topical gel was studied (**Cevc et al., 2008**).

Researchers had tried diverse approaches to either disrupt or weaken the stratum corneum to improve skin delivery using chemical edge activator or surfactants. Different surfactants such as sodium cholate and sodium deoxycholate have been integrated in the vesicular membrane, at concentration range from 20–50% mol/mol **(Elsayed and Cevc, 2011)**. In 2012, Cevc introduced the rational design

of third generation deformable vesicles with improved bilayer vesicle adaptability, stability, physical interaction and higher drug payload (**Cevc, 2012**). In recent decades, numerous dynamic pharmaceutical ingredients inclusive of thermolabile proteins, enzyme-susceptible, highly lipophilic, hydrophilic, photosensitive drugs, and high-molecular-weight molecules, have been associated with deformable vesicles (**Chen et al., 2013)**.

Ultradeformable vesicles remain successful in delivering antigenic components activating the immune response. Recently, the second generation deformable vesicles associated with tetanus toxoid successfully protected lethally challenged mice with tetanus toxin owing to Th2 responses **(Chopra and Cevc, 2014)**. With similar vesicles, burden of a difficult microbe *Mycobacterium tuberculi* causing tuberculosis was shown to be reduced when vesicle associated Ag85a was delivered non-invasively in *M.tb* spore challenged mice **(Kumar et al., 2016).** In 2017, Hussain et al., depicted the role of deformable vesicles as carrier for the transport of hydrophilic, lipophilic, thermolabile (eg, protein and peptides), and pH sensitive molecules (enzymes) or chemical sensitive candidates, toxoids, nucleic acids, and vaccines with vast appropriate outcomes **(Hussain et al., 2017**). Literature analysis suggests typical edge activators used for ultradeformable liposome preparation include sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60 and Tween 80 **(Bnyan et al., 2018**).

Over the past decades, various non-invasive routes of administration draw the substantial attention for the development of more patient-friendly dosage forms of protein antigens. The transdermal route concerns the antigen delivery across skin layers to the blood circulatory system **(Zheng et al., 2018)**. But the intrinsic properties of protein antigen such as their (i) large size, (ii) poor membrane permeation, (iii) in some instances weaker immune response, (iv) short shelf life, (v) physicochemical instability, and (vi) susceptibility to enzymatic degradation **(Bajracharya et al., 2019**) create a dreadful challenge with respect to the non-invasive delivery of antigen thus limiting their developmental and clinical application . The large size molecules with high molecular weight often result in poor membrane permeability hence low absorption **(Skwarczynski and Toth, 2020)**. Therefore, there is a need to design such formulations of optimized deformable vesicles in order to

pass through the narrow constriction (stratum corneum) via targeting the non-invasive route of administration.

2.2. COMPUTATIONAL MODELING AND SIMULATIONS OF DEFORMABLE VESICLE

The first Molecular Dynamics simulations of surfactants and lipids seemed within the 1980s after Klaus Schulten unlocks the mysteries of biology by working on first protein simulations. A decade later, molecular dynamics simulations of entirely hydrated membrane lipid bilayers have been performed. In the early 1990 numerous researchers published simulation papers on phospholipids with the recognized Berger lipid model that even became one of the leading lipid pressure fields of that time. **Tieleman et al., (1997)** first simulated the Dipalmitoylphosphatidylcholine (DPPC) liquid crystalline bilayer and studied the lipid-protein interactions. They also described simulations of other phases, lipids, and mixtures of lipids with cholesterol.

Pasenkiewicz-Gierula et al., (2000) applied a molecular dynamics simulation process to revise membrane systems at diverse levels of compositional complexity, beginning with easy lipid bilayers containing a single type phosphatidylcholine (PC) and water molecules. In last decade, researchers were highly successful in modeling and simulating the membrane system that helps in deciphering the basic features and mechanisms of fundamental processes like lipid aggregation **(Marrink et al., 2001),** vesicle fusion **(Marrink and Mark, 2003),** vesicle formation **(Vries et al., 2004),** gel and ripple phase formations **(Marrink et al., 2005),** lipid flip-flop **(Gurtovenko and Anwar, 2007),** pore formation **(Sengupta et al., 2008),** and ion permeation **(Khavrutskii et al., 2009)**. **Poger and Mark, (2010)** studied the fundamental properties of bilayers like area and volume per lipid, electron density profiles, bilayer thickness and membrane hydration using GROMOS96 force field and reproduces them experimentally with high accuracy.

Molecular Dynamics Simulation is the preferred method in understanding the base of each atomistic detail. **Cascales et al., (2012**) proposed the powerful effect of phosphatidylserine (PS) lipids on the mechanical properties of mixed phosphatidylserine- phosphatidylcholine bilayers as on boosting the PS content resulted interfacial tension and thus stiffen the bilayer.

Computational modeling is competent to revisit both the large scale and membrane level components of vesicle constitution that can only be possible with aid of a multiscale approach using models with

all atom (AA) resolution to study the membrane structure and more coarse grained (CG) models to examine the larger scale elements of vesicle structure **(Bunker et al., 2016)**. Though simulation can give in depth basic structural and dynamical data about the system, the triumph of an MD simulation is the superiority of the force field (FF) (i.e., the set of parameters dictating how the particles interact). Recently in 2016, Lyubartsev and Rabinovich reviewed the available force field for membrane simulations and proposed that Chemistry at HARvard Macromolecular Mechanics (CHARMM) and Assisted Model Building with Energy Refinement (AMBER) are the best fully AA force field parameterization for lipids **(Lyubartsev and Rabinovich, 2016).**

Zhuang et al., (2016) verify the accuracy of C36FF for miscellaneous lipid types with different head groups and tail types for both monosaturated and polyunsaturated lipids by comparing the simulation properties with experimental structural data over a wide range of temperature. Later, in continuation the phase transition property of tertiary lipid composition (saturated and unsaturated along with cholesterol) was studied through simulations using MARTINI coarse-grained force field by **(Baoukina et al., 2017)**.

Recently, 2019 has been a promising year with much advancement in membrane simulations studies via using varied algorithms and methods. **Stelter and Keyes, (2019)** apply the statistical temperature molecular dynamics (STMD) algorithm to dipalmitoylphosphatidylcholine (DPPC) vesicles of varying sizes in order to study the order–disorder gel/fluid transition. **Yang et al., (2019)** studied the effect of edge activators on the skin permeation behavior of transfersomes using the coarse-grained molecular dynamics simulations and evaluated the changes in the membrane fluidity membrane properties of vesicles caused by activators. A neoteric detailed reviewed was done by **Shi and Tian, (2019)** where they discussed recent advances in modeling and simulation along with classical simulation methods, including all-atom molecular dynamics (AAMD), coarse-grained molecular dynamics (CGMD), and dissipative particle dynamics (DPD).

3. OBJECTIVES:

The purpose of this study is to develop optimized lipid vesicles for non-invasive delivery that can cross biological barriers. The selection of lipid composition and conditions (that include physical as

well as chemical) subjected to computational simulations will contribute in optimizing non-invasive delivery of antigens. The objectives for the current study are:

1. Vesicle membrane design by molecular dynamics simulations:
    - To determine the accurate lipid-surfactant formulation for antigen delivery.
    - To study the antigen-lipid membrane interaction.
2. *In vitro* physicochemical characterization of selected specific amphipathic formulations
    - Encapsulation efficiency,
    - Vesicle size,
    - Stability of the vesicles.
3. Potential assessment of vesicle uptake by antigen presenting cells; a pre-requisite for immunogenicity for successful vesicle-mediated non-invasive delivery of antigen.

4. MATERIALS AND METHODS:

4.1. MATERIALS:

Soybean phosphatidylcholine (SPC), Surfactants (Polysorbate 80, Polysorbate 60, Polysorbate 40, and Polysorbate 20), Culture reagents, Cell lines, buffers and all other chemicals and reagents will be of analytical grade. Computational simulation analysis will rely on algorithms in Discovery Studio (Software, BIOVIA) and Gromacs.

4.2. METHODS:

The methodology for the above-mentioned objectives is as follows:

Objective 1: Vesicle membrane design by molecular dynamics simulations

Step 1: The PDB files of the lipid (*soybean phosphatidylcholine*) and surfactant (Polysorbate or Tween) will be downloaded from PubChem.

Step 2: Generation of different amphipathic formulations depending upon diverse process variables such as,

1. Choice of surfactant- Depends upon hydophile-lipophile balance (HLB) value
2. Lipid: surfactant ratio for instance; (1:1, 1:2, 1:3, 1:4)
3. Choice of Phospholipid
4. Fluid Phase

Step 3: The model membrane of different amphipathic formulations will be prepared using software like Packmol (Martinez *et al*., 2009)**.** This creates an initial start point for molecular dynamics simulations by packing molecules in defined regions of space.

Step 4: The simulations will be done using multifunctional molecular dynamics (MD) simulation package of Discovery Studio software.

Step 5: The generated PDB files of step 4 will be visualized and analyzed with the help of a DS Visualizer package of Discovery Studio software.

Step 6: Creation of PDB files of lipid membrane with model antigen to study their interaction.

Step 7: They will be simulated using molecular dynamics (MD) simulation package of Discovery Studio software.

Step 8: The generated PDB files of step 7 will be visualized and analyzed with DS Visualizer package of Discovery Studio software.

Objective 2: *In vitro* physicochemical characterization

Step 1: The stable simulated deformable vesicles formulation will be prepared according to ref. **(Cevc and Chopra, 2011)** but using Polysorbate instead of sodium cholate as surfactant. The soybean phosphatidylcholine is weighed and mixed with Polysorbate in ethanolic solution. The amphipath will further vortexed till the entire lipid completely solubilizes and then the phosphate buffer will be added. The solution will be extruded repeatedly through fine pore polycarbonate filters starting with 400 nm pore size membrane, 100 nm pore size membrane, and finally through a 50 nm pore size membrane.

Step 2: *In vitro* physicochemical characterization of the specific amphipathic formulation will be evaluated through various methods-

Encapsulation efficiency- The entrapment efficiency will be determined using vesicle leakage with time. The concentration of marker/molecule will be determined spectrophotometrically using a UV-visible spectrophotometer or fluorescence leakage estimation **(Panwar et al., 2010).**

- **Turbidity measurements-** A measurement of the turbidity as a function of wavelength using a spectrophotometer provides a means of evaluating the average size and structure of the vesicles in the dispersion. An optical Density (absorbance) spectrum is measured mainly with a double-beam

UV-Vis spectrophotometer **(Elsayed and Cevc, 2011**). This reveals the quick analysis of size of vesicles.

- **Vesicle size-** Vesicle size will be determined using dynamic light scattering (DLS) method. Amphipaths samples will be prepared in phosphate buffer, extruded through polycarbonate membranes of defined pore size and then size, zeta potential and homogeneity will be determined by using dynamic light scattering (DLS) measurements or electron microscope analysis **(Elsayed and Cevc, 2011).**
- **Stability studies-** The stability of the macromolecule/marker loaded vesicle will be evaluated after storage at −20°C, +4°C and 25°C for three months. The stability of vesicle will be determined by assessing the size and structure of vesicles over time by dynamic light scattering **(Panwar et al., 2010).** In addition to that, the leakage of the marker from the vesicles will be monitored to ensure the stability of the vesicles.

Objective 3: Potential assessment of vesicle uptake by antigen presenting cells

Step 1: Preparation of antigen loaded deformable vesicles.

The antigen-associated deformable vesicles will be prepared in accordance with objective 2 step1. As a control fluorescent dye, 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS)-encapsulated vesicles at 35 mM concentration with 10 mM Hepes buffer will be taken. The non-encapsulated dye will be removed using appropriate centricon filters **(Kessner et al., 2001).**

Step 2: Maintaining the cell culture of mammalian antigen presenting cell lines.

A murine macrophage cell line (J 774) will be used as target cells. J 774 cells will be grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Flasks seeded with freshly isolated J 774 cells will be incubated at 37^{o}C in 5% CO^2 for about 4 days until they grow nearly confluent and will be passaged by trypsinization with 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA). Passage 2 or 3 cells will be used for experiment **(Chithrani et al., 2006).**

Step 3: Incubation of cell line with optimized vesicle.

Cells will be washed with fresh media without serum and kept in the same media for 1 h before incubating with optimized vesicle (diluted with phosphate buffer saline). 0.5 ml of dilution will be

added to each dish containing 1.5 ml of media. The non-associated vesicles in solution will be washed away at the end of the incubation by rinsing three times with 3 ml of cold buffer saline.

Step 4: Detection of an optimized vesicle uptake by antigen presenting cell lines.

The pyranine (hydrophilic, fluorescent dye) assay like method will be used according to ref. **(Kessner et al., 2001).** The optimized vesicle will be prepared in 35 mM 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) solution. The keratinocytes and J774 are taken as negative and positive controls and will be transferred into plates. After cell adherence, 2 µmol of control and test vesicles will be added and incubated at either 4^{o}C or 37^{o}C. The plates will be washed and then cells are taken for absorbance assessment. To detect the absorbance changes in HPTS, its absorbance will also be analyzed.

5. Plan of Work

OBJECTIVE 1:	OBJECTIVE 2:	OBJECTIVE 3:
1. The PDB files of the soybean phosphatidylcholine and surfactants will be obtained from PubChem. 2. Model generation • Generation of file parameters • Apply random constrains and orientation • Creation of PDB files of bilayer membrane with different surfactant in varied formulations. • Visualization and analysis 3. Molecular Dynamic simulations • Apply appropriate force field • Simulation of generated PDB files • Visualization and analysis of generated simulated files 4. Analysis of interaction of model antigen with bilayer membrane • Creation of PDB files of bilayer membrane with model antigen • Simulation of generated PDB files • Visualization and analysis of generated simulated files	1. Preparation of deformable vesicles according to ref. Cevc and Chopra, (2011). 2. *In vitro* characterization of deformable vesicle through varied methods • Encapsulation efficiency (Panwar et al., 2010), • Turbidity measurements (Elsayed and Cevc, 2011), • Vesicle size (Elsayed and Cevc, 2011), and • Stabilities studies (Panwar et al., 2010) 3. Analysis of experimentally obtained data with simulated data.	1. Preparation of antigen loaded deformable vesicles. 2. Maintaining the mammalian antigen presenting cell lines culture. 3. Incubation of cell line with optimized vesicle. 4. Detection of an optimized vesicle uptake by antigen presenting cell lines (Kessner et al., 2001).

Figure: Plan of work

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